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Methods

A NEW FATHEAD MINNOW (*PIMEPHALES PROMELAS*) SUBCHRONIC TOXICITY TEST

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Abstract—The most commonly used test for evaluating the toxicity of effluents to fish and invertebrates has been the acute lethality test. However, effluents frequently are not acutely toxic, and so assessing the sublethal effects on fish and invertebrates is important. Described herein is a rapid method to estimate the chronic toxicity of effluents to the fathead minnow (*Pimephales promelas*). Tests are initiated using newly hatched minnow larvae and are run for 7 d under static-renewal conditions. It is a cost-effective, simple, short-term toxicity test, and survival and growth are the measures of effects. Approximately 70 tests using this technique have been run with various types of effluents. Included in this article are results from representative effluent tests and also from tests conducted using Dursban, zinc and copper. The results indicate that this subchronic toxicity test gives a good estimate of chronic toxicity.

Keywords—Effluents *Pimephales promelas* Sublethal effects Toxicity test Zinc Copper Dursban

INTRODUCTION

The use of early life stage (ELS) tests and life cycle chronic tests has been emphasized increasingly in the evaluation of sublethal effects of toxicants to fish. The embryonic and larval stages have been identified as the most sensitive to toxicants [1,2]. Macek and Sleight [2] reviewed life cycle chronic tests and reported that in most cases the estimates of the application factors derived from exposure of the early life stages reasonably approximated the maximum acceptable toxicant concentration (MATC) determined in life cycle chronic toxicity studies. The cost, time and effort required for an ELS test are much less than for life cycle chronic tests, although the economic constraints and time required to conduct ELS tests still do limit the number of tests that can be done. While most ELS tests have been conducted with single toxicants, limited ELS testing with multiple toxicants [3,4] or effluents [5,6] has been done. Testing of industrial and municipal effluents has primarily been by means of acute toxicity tests, but many industrial effluents are not acutely toxic [7]. Because conducting effluent ELS tests on-site is quite costly and the shipping of effluents for flow-through testing not practical, a method to test for the chronic effects of effluents is needed.

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The fathead minnow *(Pimephales promelas)* is currently the most commonly used species for ELS tests, for many reasons. It has a widespread distribution, is an important forage fish in the food chain and can be cultured readily in the laboratory. A large toxicity database has been established for acute, partial and life cycle chronic tests using the fathead minnow. The general rationale for using the embryo and larval stages has been discussed previously [1,2,8,9]. In a fathead minnow life cycle test using linear alkylate sulfate the newly hatched fathead minnow larvae were found to be the most sensitive of all stages [10]. In each ELS test with four organic chemicals, Benoit et al. [9] found larval growth to be the most sensitive measure of the chemical's effect, with survival equally or less sensitive than growth; no toxic effect was produced in the embryonic development and hatching period. Most toxicity to the developing larvae appears in the first few days post-hatch, and the heaviest mortality frequently occurs in the first week or two post-hatch [5,9,11,12]. Increased sensitivity of minnow embryos was observed only when eggs were exposed to zinc prior to water hardening, which occurs 30 to 60 min after spawning [13].

This article suggests a static-renewal test using growth and survival of newly hatched fathead minnow larvae as a rapid method to assess the subchronic effects of effluents and chemicals. The

need for such a test is apparent [14,15] for the estimation of the effluent dilution that will not cause impairment. This renewal test method, designed for effluent testing, is useful when space is a problem, sample volumes are limited and the cost of conducting tests is a factor, and when several discharges must be tested simultaneously, which makes flow-through testing impractical.

TEST PROCEDURE

The test chambers for the fathead minnow larval growth test are 30.5 cm long × 15.2 cm wide × 10.2 cm high. Each test chamber is subdivided by glass partitions into four compartments, each 12.7 cm long × 7.6 cm wide × 10.2 cm high. A 30.5 cm × 10.2 cm piece of 60-mesh stainless steel screen is glued across the end of each partition. The edge of the screen is glued to the bottom, and the end of the screen is also glued to the sides of the test chamber. This design (Fig. 1) provides a 1-in sump area where water can be added and removed while the fish in each compartment are left relatively undisturbed. New test solution is added slowly into the sump areas. Two liters of test water are used per test chamber, with 10 fish in each of the four compartments. A water connection exists between compartments but little water movement occurs unless the test solution is being added or removed. The test is run for 7 d, and each day the test solution is renewed to maintain satisfactory dissolved oxygen levels. The test is run at 25 ± 2°C and utilizes a 16-h photoperiod. The techniques employed in this method are similar to those suggested by Benoit et al. [9] and the U.S. Environmental Protection Agency (EPA) [8]. With the continued use of this method for effluent testing, we have found it beneficial to reduce the water volume in the test chambers to 1 liter. The chambers are easily siphoned, as described above, with the lesser volume. One liter gives a much better surface-to-volume ratio, so when effluents with high biological oxygen demand are tested, the oxygen concentrations do not drop too low.

Newly hatched fathead minnow larvae, preferably less than 24 h old, are used to begin a test. When using more than one spawning to begin a test, the larval fish from all spawnings are combined. Larvae are randomly assigned, using a large-bore pipette, to all compartments and treatments until 10 larvae are in each compartment, 40 per treatment. The amount of water added to the test water when putting larvae into chambers should be kept to a minimum to avoid dilution of the test concentrations. The fish are fed live brine shrimp nauplii (*Artemia salina*), less than 24 h old, that have been rinsed with fresh water. Since larvae begin feeding during the first day of hatch, feeding should be started immediately, and the brine shrimp nauplii must be small enough for the fathead larvae to ingest. Growth rates depend on the brine shrimp nauplii used, since different strains of the cysts can vary in nutritional adequacy [16]. The volume of water added when shrimp are fed should also be kept to a minimum. Live shrimp should be available during the entire daylight period. Excess amounts of shrimp should be avoided because the decay of brine shrimp causes dissolved oxygen levels to decrease. The U.S. EPA [8] recommends three feedings a day, 4 h apart, for newly hatched fish for the first week, and the same procedure is recommended for this test method.

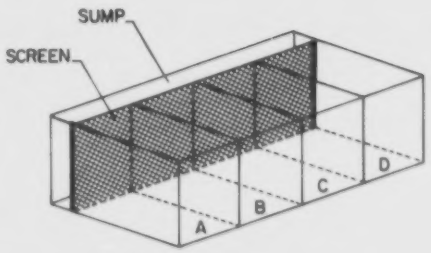

Fig. 1. Test chamber for static-renewal fathead minnow larvae growth test. Chamber has four compartments with a 60-mesh screen across the ends of the partitions, and a 1-in sump area that facilitates renewal of test solution.

During renewal of test solutions, each compartment bottom is carefully siphoned to remove the dead brine shrimp. A Tygon foot modified from a Tygon U-tube is attached to the siphon hose and used to loosen particulate matter. When each compartment has been cleaned, additional water can be removed from the sump without danger of removing larvae. The water level in the test chamber is lowered to at least 7 to 10 mm, leaving an adequate volume in which the larvae can swim. The test solution is added slowly to the sump to avoid disturbing the larvae. Setting the test chambers on a light box makes counting, observing and siphoning the compartments easier. As recommended in the test guidelines of the U.S. EPA [8], the test solution should be siphoned into a white tray to allow ready observation of any larvae inadvertently siphoned. Daily survival and any swimming or developmental abnormalities should be recorded. As with the test methods for life cycle and ELS tests, for an acceptable test, the average mortality in the control should not exceed 20%.

At test termination, the live fish are counted,

dried and weighed, or preserved in 4% formalin for drying and weighing at a later data. Fish can be dried and weighed without the intermediate step of preservation with equal success. Four groups of 10 larvae each can be randomly selected and preserved at the start of the test to obtain initial weights, if desired. This is useful when comparing percent weight gain when tests are repeated or when using fish older than 24 h. All preserved fish are rinsed in distilled water to remove any formalin residue and minimize weighing errors. They are then transferred into preweighed aluminum weigh-boats, which can be nested during drying without the bottoms of the boats touching any fish in the next weigh-boat, allowing more samples to be dried simultaneously. The fish are dried at 100°C for a minimum of 2 h. Fish have been dried for up to 18 h and the comparison of the weights at intervals after 2 h yielded the same results (T. Roush, personal communication, Environmental Research Laboratory–Duluth). Immediately upon removal from the drying oven, the weigh-boats are placed into a desiccator to prevent absorption of moisture from the air until larvae are weighed. Weighing must be accurate to 1/100 milligram. Mean weights are determined by dividing the group weight by the actual number of larvae weighed.

The data on the four group dry weights for each treatment can be statistically analyzed using a variety of standard techniques. Even though the four compartments are connected, the assumption is made that they behave as replicates.[1] The analysis assumes that the variability in mean treatment response is inversely proportional to the number of measurements (or fish) in the treatment. The analysis is performed using MINITAB (copyright by Pennsylvania State University, 1982) by estimating a *t* statistic for comparing mean treatment and control responses, using a weighted regression with weights equal to the number of measurements in the treatments. The *t* statistic is then compared with the critical *t* statistic for the standard Dunnett's test [17]. The survival data are arcsine-transformed (a variance-stabilizing transformation) before the regression analysis. Benoit [18] describes methods used for chronic tests. Also, the American Society for Testing and Materials [19] and the U.S. EPA [8] both discuss in detail the statistical analyses of such data.

[1]In recent effluent studies, the assignment of treatments to random positions has been done daily. This alleviates much of the concern connected with having the replicates clustered. However, in cramped working space, such as mobile laboratories, the logistical problems of handling the test chambers are aggravated. After discussing this with the statistician, we intend to use two replicates of 10 fish for each treatment. These are to be completely separate so that the two replicates can be randomized with respect to test position. This way the positions do not need to be rerandomized each day and many of the logistical problems are eliminated. The statistical opinion is that two replicates that can be randomly positioned are better than four, clustered replicates.

RESULTS

To date, the subchronic test has been used primarily for effluents, with a high success rate. At this time, about 70 tests have been completed using a variety of effluent types, such as refinery, metal plating, chemical manufacturing and pulp and paper plant effluents. Results from representative tests with effluents and with single chemicals are presented here. Table I shows the mean weight and survival at 7 d of fathead minnow larvae exposed to a fertilizer plant and two different refinery effluents plus water samples taken from the receiving water below the discharges of the fertilizer plant and refinery No. 2. The ambient toxicity tests involved measuring the toxicity of samples collected from the receiving water to estimate the die-away of toxicity and to obtain an estimate of an effluent's persistance. The mean group weights are the actual values for each replicate and the overall treatment mean is a weighted average of the replicate means. The mean weights vary slightly in these tests and the weights obtained in the refinery No. 1 test are slightly lower than for the other tests. This was attributed to lower mean test temperatures during the on-site test with refinery No. 1 effluent. Even so, the mean coefficient of variation was less than 5% in that test. The initial weights of the groups of larvae used were obtained, and an average sixfold increase in weight in 7 d was observed. Growth of the fish in water from Stations 3 and 5, collected below refinery No. 2 and below the fertilizer plant, respectively, was reduced to a level comparable to the 10% effluent dilution test result. Yet fish weights in the ambient samples from 10 miles downstream did not show growth reductions.

Comparison of two diluent waters with the effluents for two coke plants is shown in Table 2. No differences between river water and well water were observed for either effluent. Growth was the most sensitive measure of effects for both effluents in both dilution waters. A sixfold increase in weight was observed in 7 d and a difference as small as 0.12 mg was significant. The subchronic effluent tests described herein have been conducted using seven 24-h composite samples (one for each day), one 24-h composite sample (renewed daily),

Table 1. Seven-day growth and survival of fathead minnow larvae exposed to three effluents, and to receiving water samples taken from the stream where effluents were discharged

Effluent or stream sample	% effluent concentration (v/v) or station number	Weighted mean[a] (mg)	Standard error	Mean percent survival[a]
Refinery No. 1	Control	0.346	0.019	95
	1	0.445	0.019	98
	5	0.404	0.020	90
	10	0.329	0.022	75
	50	0.132[b]	0.054	13[b]
	100	—[c]	—	0[b]
Fertilizer plant	Control	0.608	0.019	91
	1	0.589	0.027	98
	3	0.600	0.028	90
	10	0.497[b]	0.029	85
	30	0.297[b]	0.030	78
	100	—[c]	—	0[b]
Refinery No. 2	Control	0.608	0.018	91
	1	0.599	0.026	93
	3	0.562	0.026	93
	10	0.418[b]	0.026	90
	30	0.276[b]	0.038	43[b]
	100	—[c]	—	0[b]
Ambient stations				
Upstream control	1	0.608	0.024	91
	2	0.646	0.033	95
Below refinery No. 2	3	0.494[b]	0.034	93
	4	0.525	0.036	83
Below fertilizer plant	5	0.429[b]	0.040	45[b]
	5A	0.670	0.033	95
	6	0.627	0.035	85
	7	0.728	0.035	83
	8	0.650	0.033	94
	9	0.762	0.035	85

[a]Mean values of four groups of fish weighed per concentration.
[b]Significantly different from control ($p \leq 0.05$; analysis of variance with Dunnett's test). Survival data arcsine-transformed prior to analysis.
[c]All fish died prior to 7 d.

or seven daily grab samples. Other tests have also been conducted using one grab or one composite effluent sample that was shipped to Environmental Research Laboratory-Duluth.

Three single-toxicant, 7-d subchronic growth tests were conducted with zinc, copper and Dursban[2] in Lake Superior water (Table 3). No zinc-induced growth inhibition was observed in 7 d at 184 μg/L, while survival was significantly lower at that concentration. Copper and Dursban had the lowest effect concentrations based on growth. Coefficients of variation of the control weights are all less than 7%; in the majority of all tests conducted, they were less than 4%.

Comparisons of chronic effect limits and estimated MATC values from the 7-d subchronic growth tests and the 32-d ELS, partial or life cycle chronic test MATCs done in the same or similar water are given in Table 4. The MATC is the hypothetical toxic threshold concentration between the no observed effect concentration (NOEC), which causes no adverse effect, and the lowest observed effect concentration (LOEC) that causes an adverse effect [20]. Benoit et al. [8] restated the definition of MATC as an "estimated" MATC if it is calculated from test data that do not involve all the developmental stages in a toxicity test. An

[2]Dursban is the Clark Granular Larvicide Formulation. The trade name is used for identification only, as the U.S. EPA neither recommends nor endorses commercial products.

Table 2. Seven-day growth and survival of fathead minnow larvae exposed to two coke plant effluents and two types of dilution water

Type of effluent	% effluent (v/v)	Dilution water	Mean group weights				Overall weighted mean (mg)	Standard error	Overall mean percent survival
			A	B	C	D			
Coke plant No. 1	Control	River water	0.52	0.56	0.49	0.54	0.527	0.026	85
	0.5		0.39	0.55	0.47	0.43	0.464	0.025	93
	1		0.39	0.44	0.34	0.40	0.392*	0.027	80
	5		—	—	—	—	—	—	0*
	10		—	—	—	—	—	—	0*
	50		—	—	—	—	—	—	0*
Coke plant No. 1	Control	Well water	0.50	0.51	0.59	0.48	0.521	0.024	98
	0.5		0.46	0.48	0.48	0.49	0.477	0.025	90
	1		0.44	0.40	0.46	0.29	0.404*	0.026	85*
	5		—	—	—	—	—	—	0
	10		—	—	—	—	—	—	0
	50		—	—	—	—	—	—	0
Coke plant No. 2	Control	River water	0.56	0.76	0.62	0.63	0.643	0.036	100
	1		0.70	0.74	0.79	0.63	0.709	0.038	90
	5		0.78	0.72	0.66	0.61	0.693	0.036	100
	10		0.46	0.71	0.59	0.60	0.593	0.037	98
	50		0.31	0.35	0.33	0.33	0.330*	0.038	90
	100		0.19	0.14	0.09	0.18	0.156*	0.045	40*
Coke plant No. 2	Control	Well water	0.50	0.51	0.59	0.48	0.521	0.029	98
	1		0.57	0.59	0.73	0.72	0.651	0.029	98
	5		0.58	0.67	0.66	0.57	0.623	0.030	88
	10		0.51	0.60	0.67	0.56	0.587	0.029	98
	50		0.34	0.36	0.39	0.46	0.387*	0.029	95
	100		0.19	0.14	0.09	0.18	0.156*	0.045	40*

*Significantly different from the control ($p \leq 0.05$), with survival data arcsine-transformed prior to analysis.

MATC can be calculated by taking the geometric mean of the upper (LOEC) and lower (NOEC) chronic effect limits from a chronic test [21]. As Table 4 shows, the 7-d tests gave values very comparable to the literature values; in fact, the upper and lower limits overlap. Based on the results of the subchronic growth test, the toxicity threshold of 7 d appears to be in good agreement with the chronic values in both partial and complete life cycle tests.

Comparative data for 96-h and 7-d LC50s plus their confidence intervals, lower and upper chronic limits and the calculated MATCs are presented in Table 5 for both effluents and the two metals. The LC50s were computed by the trimmed Spearman-Karber method [22] using larval mortality data during the 7-d growth test. The chronic values were lower than either the 96-h or 7-d LC50s for eight of the nine tests and on the average were fourfold lower. These results indicate that the effects on growth give a lower estimate of chronic toxicity even at 7 d than does measuring only mortality at 7 d. However, both the effects on survival and growth should be used in evaluating the chronic effects of effluents or chemicals.

DISCUSSION

The variability of toxicity test data with effluents is less than that of effluent and receiving water flows [23]. Because many effluents have LC50 values that are close to or greater than 100%, assessing subchronic effects of effluents is important, since dilution in the receiving water may not be sufficient to prevent chronic toxicity impact in the receiving body of water. The results of some ELS, partial and life cycle chronic fathead minnow effluent tests have shown growth to be a more sensitive indicator of toxic stress than survival [5,24]. Conducting longer-term life cycle or ELS tests is not cost effective, since testing must be done on-site to include the variability in effluents. Recently, emphasis has been placed on controlling the toxicity of effluents in addition to that of specific constituents. This 7-d subchronic test is useful in estimating the chronic toxicity of

Table 3. Effects of zinc, copper and Dursban on the 7-d growth of larval fathead minnows in Lake Superior water (hardness, 48 mg/L as $CaCO_3$)

Chemical	Mean measured concentration (μg/L)	Overall weighted mean[a] (mg)	Standard error
Zinc	Control	0.600	0.026
	16.4	0.738	0.027
	38.7	0.708	0.028
	84.6	0.697	0.027
	184[b]	0.522	0.030
	374	0.300[c]	0.097
Copper	Control	0.463	0.019
	6.06	0.580	0.018
	9.89	0.480	0.018
	25.8	0.259[c]	0.020
	38.1	0.219[c]	0.021
	75.2	0.095[c]	0.026
Dursban[d]	Control	0.503	0.045
	0.81	0.406	0.038
	1.6	0.479	0.039
	3.7	0.474	0.040
	7.4	0.251[c]	0.041
	12.0	0.244[c]	0.047

[a]Overall weighted mean is the individual fish weight based on number of fish weighed per treatment.
[b]Effect concentration for zinc based on survival data analysis.
[c]Significantly different from control $p \leq 0.05$; analysis of variance with Dunnett's test).
[d]Data based on three replicates.

effluents quickly and cost-effectively, and with good sensitivity, based upon available comparisons with other chronic data.

The renewal method offers two important advantages for effluent testing. Many effluents have variable compositions and toxicities. In the test described, a new sample of effluent can be used at each daily renewal, thereby providing a more representative effluent exposure than if a single effluent sample is used. Second, there often are multiple discharges close together that must be tested simultaneously to determine the effect of each as a part of the total combined effect. These renewal tests are much more practical to do simultaneously than are flow-through tests because different dilution waters can be used for each, whereas for flow-through test systems the logistics usually require that only one dilution water be used. However, if it is necessary to do flow-through testing, slight changes in the test procedure can be made to allow this. The cost of the test is much less than that of ELS tests, as the time needed for one test with five concentrations and a control is 10 to 14 total working hours, excluding analyses.

SUMMARY

The subchronic test using the fathead minnow described allows more tests to be conducted than can be done if the more time-consuming ELS tests are used. The test also incorporates standard procedures developed for such tests [8,9] wherever possible. Considering the limited data available from life cycle chronic tests, and the effort and cost required to conduct ELS tests, the shorter-term, and therefore lower-cost, larval growth and survival test provides a means of obtaining an estimate of the impact of a complex effluent or single toxicant on growth, with possible application to

Table 4. Comparison of the 7-d subchronic growth test with the 32-d ELS test and partial or complete life cycle tests with fathead minnows

	Effect limits		Estimated MATC or measured MATC	Reference
	Lower	Upper		
Copper				
7-d subchronic test	9.9	26	16	This study
32-d ELS test	13	26	19	25
Complete life cycle test[a]	11	18	14	26
Dursban				
7-d subchronic test	3.7	7.4	5.2	This study
32-d ELS, encapsulated formulation	2.2	4.8	3.2	27
32-d ELS, technical-grade	1.6	3.2	2.3	27
Zinc				
7-d subchronic test	85	130	125	This study
Partial life cycle test	30	180	74	28
Complete life cycle test	78	145	106	13

[a]Test was conducted in water with 30 mg/L (as $CaCO_3$) hardness rather than 48 mg/L (as $CaCO_3$) for other copper tests.

Table 5. Comparison of 96-h and 7-d LC50s for larval fathead minnow survival and 7-d effect levels for larval weights

Effluent or chemical[a]	96-h LC50	95% confidence limits on 96-h LC50	7-d LC50	95% confidence limits on on 7-d LC50	7-d effect limits		Calculated MATC
					Lower	Upper	
Refinery No. 1	50	39–64	17	14–22	10	50	22.4
Coke plant No. 1							
River water	2.1	2.0–2.3	1.8	1.5–2.1	0.5	1	0.7
Well water	2.2	—[b]	1.9	1.6–2.3	0.5	1	0.7
Coke plant No. 2							
River water	—[c]	—[c]	87	73–100	10	50	22.4
Well water	—[c]	—[c]	88	75–100	10	50	22.4
Fertilizer plant	42	36–50	33	26–42	3	10	5.5
Refinery No. 2	47	40–54	26	20–33	3	10	5.5
Zinc[d]	238	211–269	238	211–269	85	184	125
Copper	—[c]	—[c]	70	48–104	9.9	26	16.0

[a]Effluent values expressed as percents; metal concentrations expressed as μg/L.
[b]Confidence interval not reliable by trimmed Spearman-Karber method.
[c]Mortality was <50% at 96 h.
[d]Zinc limits based on significant difference ($p \leq 0.05$) for survival.

estimating reproductive success. These tests, as well as those cited before [9,10], indicate that larval growth is among the most sensitive measures of toxic stress and that this test can be used to estimate the NOECs. It can be used when an estimate of acute toxicity is not a sensitive enough measure. Results obtained with this 7-d test method demonstrate the usefulness and consistency of such tests for rapid evaluation of sublethal effects of complex effluents and of single toxicants.

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